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(21) International Application Number: PCT/US99/29405 (22) International Filing Date: 10 December 1999 (10.12.99) (30) Priority Data: 09/210,330 11 December 1998 (11.12.98) US (71) Applicant: CLONTECH LABORATORIES, INC. [US/US]; 1020 East Meadow Drive, Palo Alto, CA 94303 (US). (72) Inventors: LUKYANOV, Sergey Anatolievich; ul. Golubinskaya 13/1-161, Moscow (RU). FRADKOV, Arcady Fedorovich; ul. Dnepropetrovskaya, 35/2-14, Moscow, 113570 (RU). LABAS, Yulii Aleksandrovich; ul. Generala Tyuleneva, 35-416, Moscow, 117465 (RU). MATZ, Mikhail Vladimirovich; ul. Teplii stan, 7/2-28, Moscow, 117465 (RU). (74) Agent: ADLER, Benjamin, A.; McGregor & Adler, 8011 Candle Ln., Houston, TX 77071 (US).		(81) Designated States: JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF (57) Abstract The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.		

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**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
USES THEREOF**

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, methods of identifying the DNA sequences encoding the proteins and
15 uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for
20 marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps,
25 however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or
5 substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

10 Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing
15 subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by
20 Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets
25 in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence
5 encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is
10 provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid
15 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a
20 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the
25 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

Figure 2A shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246-1251 (1996)). Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

5 Figure 6 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

10 Figure 8 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

15 Figure 11 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

25 As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., *Nature* 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for

expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

15 A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

25 A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination
5 sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

10 The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that
15 provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining
20 the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a
25 transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by
5 exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to
10 eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter
15 cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an
20 identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example,
25 heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

5 The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: 10 serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine; X: any residue). NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552- 15 59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

20 In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group 25 consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid
5 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a
10 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the
15 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an
20 intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1**Biological Material**

- 5 Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

TABLE 1**Anthozoa Species Used in This Study**

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp.	Western Pacific	green spots on oral disk

"green"		
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2**cDNA Preparation**

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthesized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG(T)₁₃
(SEQ ID No. 17)

10

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
15 5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

20

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCGrGrGrG
(SEQ ID No. 53)

25

EXAMPLE 3Oligo Design

To isolate fragments of novel fluorescent protein cDNAs,
5 PCR using degenerate primers was performed. Degenerate primers
were designed to match the sequence of the mRNAs in regions that
were predicted to be the most invariant in the family of fluorescent
proteins. Four such stretches were chosen (Table 3) and variants of
degenerate primers were designed. All such primers were directed to
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2
shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used
for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5) GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4Isolation of 3'-cDNA Fragments of nFPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 μ l of 20-fold
 10 dilution of the amplified cDNA sample was added into the reaction
 mixture containing 1X Advantage KlenTaq Polymerase Mix with
 provided buffer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel
5 fluorescent protein cDNAs, two nested 5'-directed primers were
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were
then amplified using two consecutive PCRs. In the next PCR reaction,
the novel approach of "step-out PCR" was used to suppress background
amplification. The step-out reaction mixture contained 1x Advantage
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer
(CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer
(see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of
T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the
amplified cDNA sample in a total volume of 20 μ l. The cycling profile
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was diluted 50-fold in water and one μ l of this dilution
was added to the second (nested) PCR. The reaction contained 1X
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),
20 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M
of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was then cloned into pAtlas vector (CLONTECH) according
25 to the manufacturer's protocol.

TABLE 5Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of nFPs in *E. coli*

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 6). Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1 µl of 10 the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene 15 Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard 20 protocols.

25 All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl

of the overnight culture was transferred into 200 ml of fresh IB medium containing 100 μ g/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The
5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaaca agttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacattttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgagggttggaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgtccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaac (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcaacttaagaagaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7**Novel Fluorescent Proteins and cDNAs Encoding the Proteins**

Seven cDNA full-length cDNAs encoding fluorescent
5 proteins were obtained (SEQ ID Nos. 45-51), and seven novel
fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral
properties of the isolated novel fluorescent proteins are shown in Table
7, and the emission and excitation spectra for the novel proteins are
shown in Figures 3-11.

10

TABLE 7Spectral Properties of the Isolated NFPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

5 *relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

**relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two
5 proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β -sheets are underlined; the residues whose side
10 chains form the interior of the β -can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
- 15 2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al., (1990) J. Biol. Chem. 265: 11823-11826.
- 20 7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification
25 are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, 5 molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope 10 of the claims.

WHAT IS CLAIMED IS:

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

5 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

10

2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer
15 selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

3. A method of analyzing a fluorescent protein in a cell,
20 comprising the steps of:

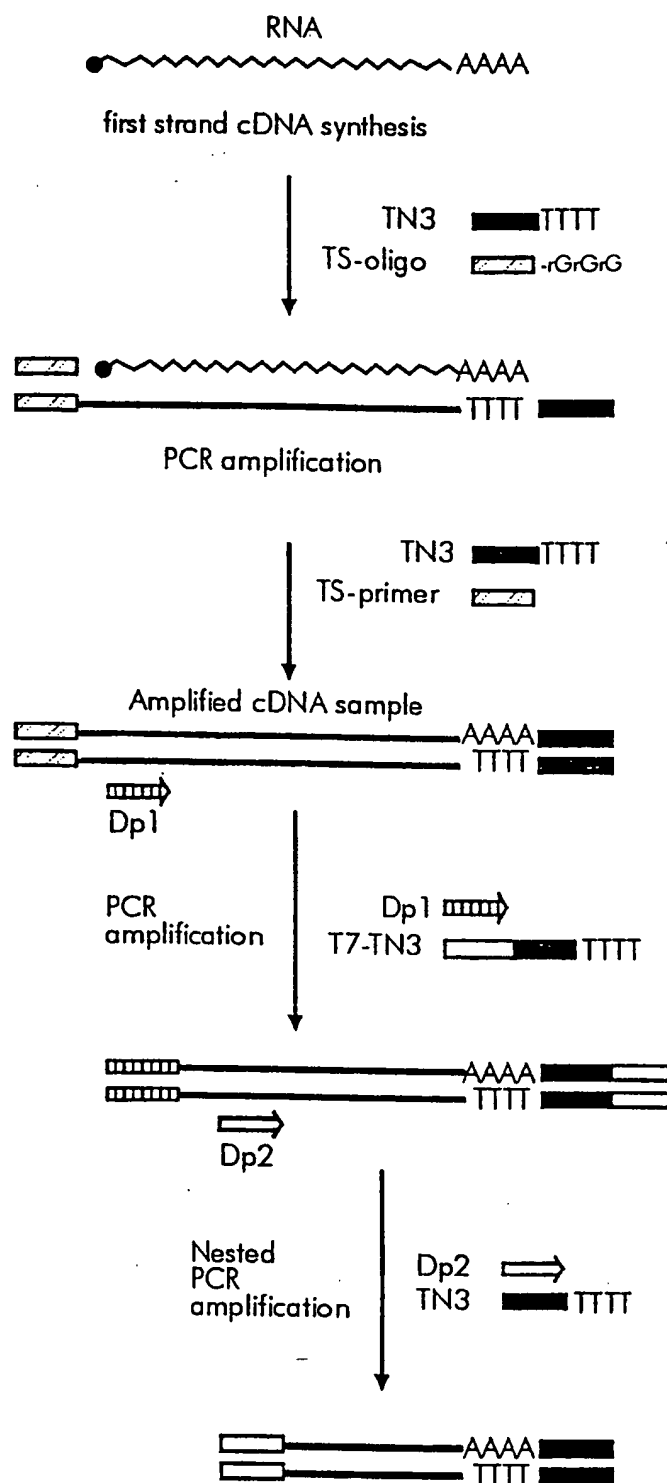
a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63;
and

25 b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:

sorting said cell according to said signal.

5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.
- 5 6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.
- 10 7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.
8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.
- 15 9. The method of claim 8, further comprising the step of:
 identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of
20 interest.
10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.



Cloning

FIG. 1

1/11

SYNTHESIS OF A cDNA LIBRARY

10	20	30	40	50	SEQ ID#
MSKGEELFTG.VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTLKFICTT.GKLPVP..W GFP					54
MAQSKHGLTK.EMTMKYRMEGCVDGHKFVITGEGIGYPFKGKQAINLCVV..EGGPLPFAE					57
--H-----KE.-----H-----N-----T-----I-----S-					58
MSWSKSVIKE.EMLIDLHLEGTfNGHYFEIKGKGKGPNEGNTNTVTLEVT..KGGPLPFGW					59
...M-AL---Y-K-N-TM--VV--LP-K-R-D-----YQ-SQEL--T-V.....SY					62
-RS--N-----F-RFKVRM--V---E---E-E-E-R-Y--H---K-K--.....A-					60
M-C--N-----F-RFKVRM--V---E-----E-E-R-Y--HCS-K-M--.....AF					63
...MASFLKK.TMPFKTTIEGTVNGHYFKCTGKGEGNPFEGTQEMKIEVI..EGGPLPFAF					61
MALSNKFIGD.DMKMTYIIMDGCVCNGHYFTVKGEKNPKPYEGTQTSTFKVTMANGGPLAFSF					55
ⓀALTTMGVIKPDMMKIKLMEGNVNGHAFVIEGEGEGKPYDGTHTLNLEVKMAEGAPLPFSY					56
60	70	80	90	100	110
PTLVTTFSYGVQCFsRYPDHMKQHDFKsAM...PEGyVOERTIFFKDDGNYKTRAEVKFEgd..					GFP
DILSAAFNYGNRVFTEYPQDIV..DYFKNSC...PAGYTWDRSFLFEDGAVCICNADITVSVEEN					zFP506
-----G-K--D-I-----G-----V-----K--					zFP538
HILCPQFQYGNKAFVHHPDDIP..DYLKLSF...PEGYTWERSMHFEDGGLCCITNDISLTGN..					dsFP483
D--TTM-----R---NY-E---..-IF-QTCsGPNg--S-Q-T-TY---V-TA-SN--VV-D..					dgFP512
D--S-----S-VY-K--A---..-K-----FK---V-N-----VVTV-Q-S--QDg..					drFP583
D--S-----S-VY-K--A---..-K-----FK---V-N-----VVTVSQ-S--KDG..					dmFP592
HILSTSCMYGSKTFIKYVSGIP..DYFKQSF...PEGFTWERTTTTYEDGGFLTAHQDTSLDGD..					asFP600
DILSTVFKYGNRCFTAYPTSMp..DYFKQAF...PDGMSYERTFTTYEDGGVATASWEISLKGN..					amFP486
DILSNAFQYGNRALTKYPDDIA..DYFKQSF...PEGYSWERTMTFEDKGIVKVKSDISMEED..					cFP484
120	130	140	150	160	170
TLVNRIELKGIDFKEDGNILGHKLEYNYNshNVYIMADKQKNGIKVNFKIRHNIEDGSVQL					GFP
CMYHESKFYGVNF PADGPVM.KKMTDNWEPsCEKIIPV PKQGI LKGDVSMYI.LLKDGGRLR					zFP506
-I--K-I-N-M-----T--A-----M-----Y-					zFP538
CENYDIKFTGLNFPPNGPVV.QKKTtGWEpSTERLYP..RDGVLIGDIHhALTVEGGGHYV					dsFP483
T-----H-M-A---LD--MM.--R-MK-----IMFE ---L-R-D-AMS-LLK-----R					dgFP512
--I-KV--I-V---SD---M-----M--A-----..-K-E--K--KLKD---L					drFP583
--I-EV--I-V---SD---M--RR-R-----S-----..-K---M--RL-----L					dmFP592
CLVYKVKILGNNFPADGPVM.QNKAGRWEpATEIVYE..VDGVLRGQSLMALKCPGGRHLT					asFP600
CFEHKSTFHGVNF PADGPVM.AKKTtGWDpSFEKMTV..CDGILKGDVTAFLMLQGGGNYR					amFP486
SFIYEIRFDGMNFPPNGPVV.QKKTlKWEpSTEIMYV..RDGVLVGDISHSLLLEGGGHYR					cFP484
180	190	200	210	220	230
ADHYQQNTPIGDG.PVLLPDNHYLSTQSALS KDPNEKRDMVLLFVTAAGITHGMDELYK					GFP
CQFDTVYKAKSV..PRKMPDWHFIQHKLtREDRSdAKNQKWLtEHAIASGSALP					zFP506
-----S---E-----L-----Q-----FP---A					zFP538
CDIKTVYpAKK...PVKMPGYHYVDTKLVIRsNDKEFM.KVEEHEIAVARHHPLOsQ					dsFP483
--FE-I-KPN- V-----D--F--HYIE-T-QQNYYN V--LT-V-E--YSS-EKIGKSKA					dgFP512
VEF-SI-M---..-QL---Y---S--D-T-HNEDYT.I--QY-RTEG---LFL					drFP583
VEF-SI-MV-- PS-QL---Y---S--DMT-HNEDYT V--QY-KTQ-----FIKPLQ					dmFP592
CHLHTTYRSKKPASALKMPGFHFEDHRIEIMEEVEK GK.CYQYEAAGVRYCDAAPSKLGHN					asFP600
CQFHTSYKTKK...PVTMPPNHVVEHRIARTDLdKGGN.SVQLTEHAVAHITSVFPF					amFP486
CDFKSIYKAKK...VVKLPDYHFVDHRIEILNHdKdYN.KVTLYENAVARYSLLPSQA					cFP484

FIG. 2A

»

MKCKFVFCLSFLVLAITNANI FLRNEADLEEKTLRIP

FIG. 2B

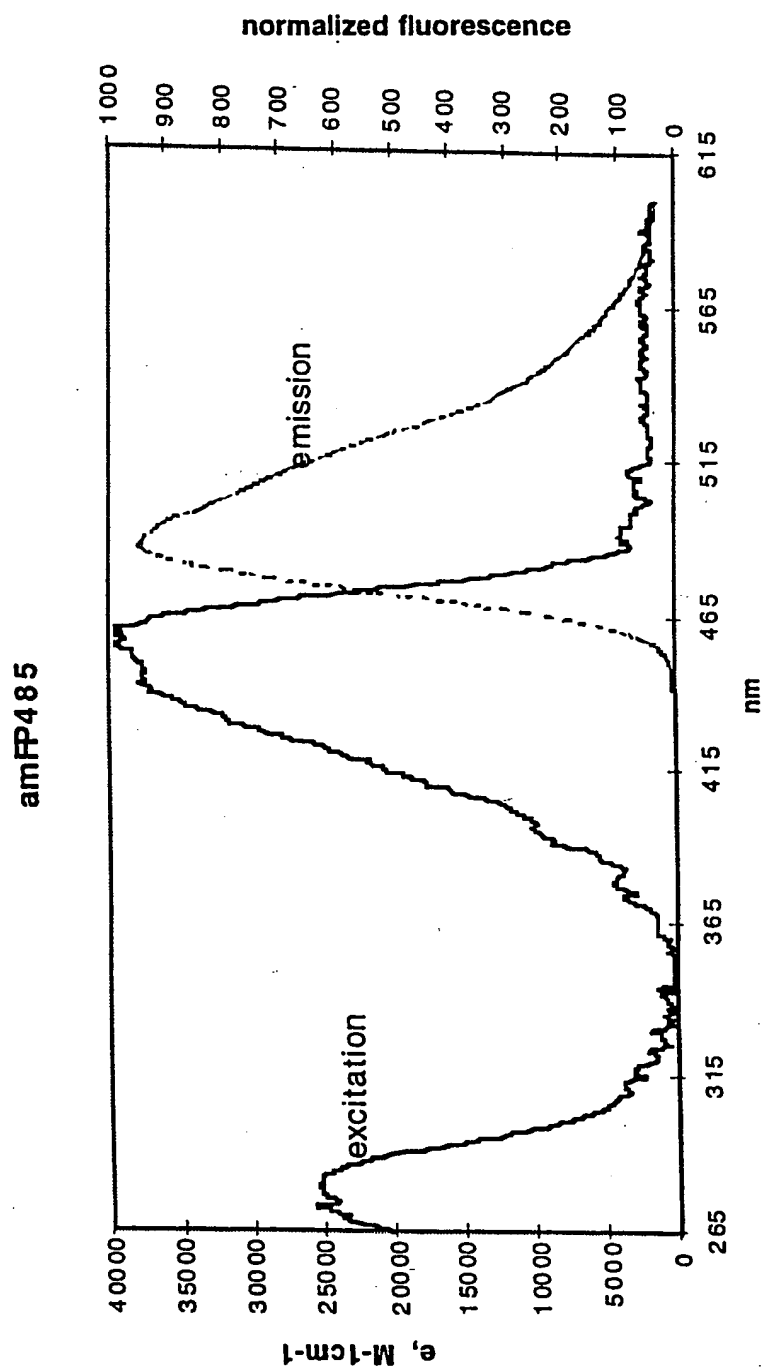


FIG. 3

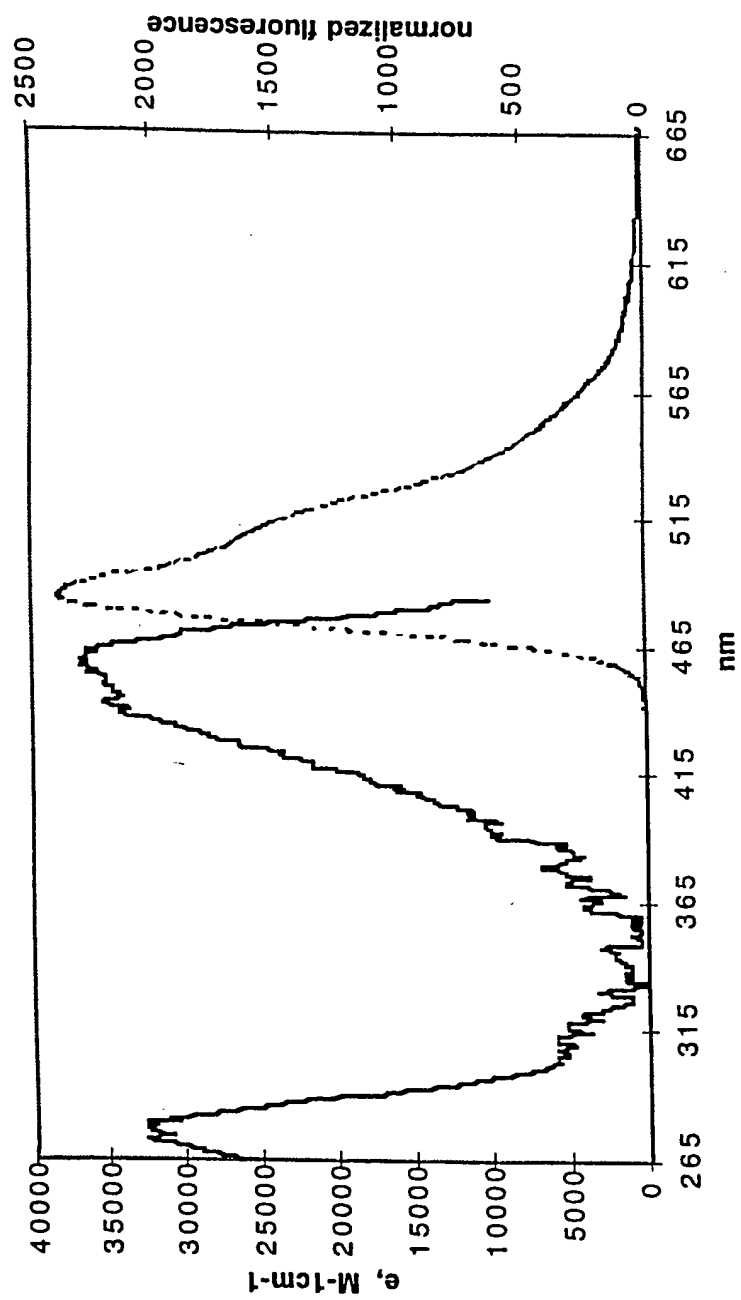


FIG. 4

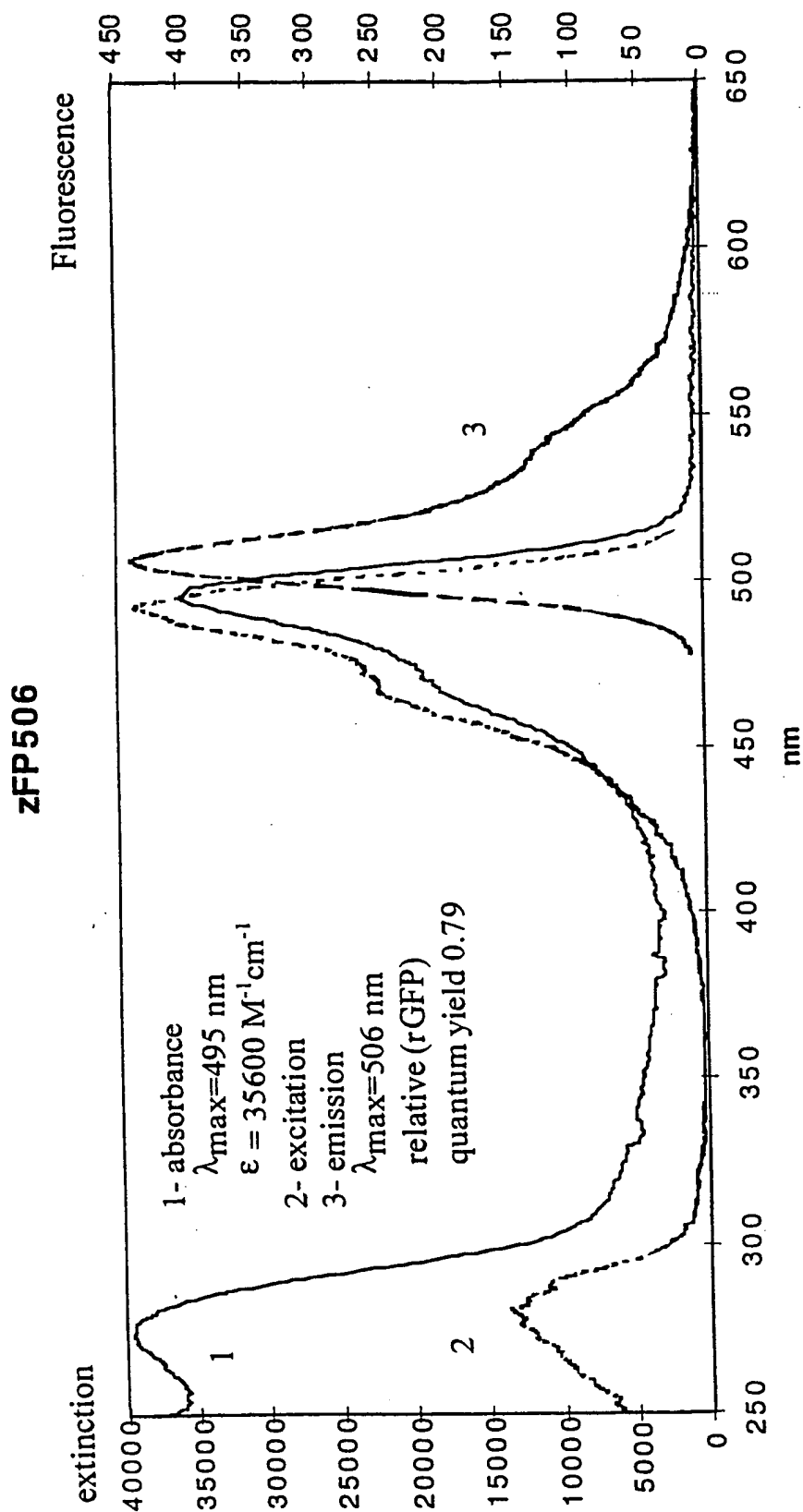


FIG. 5

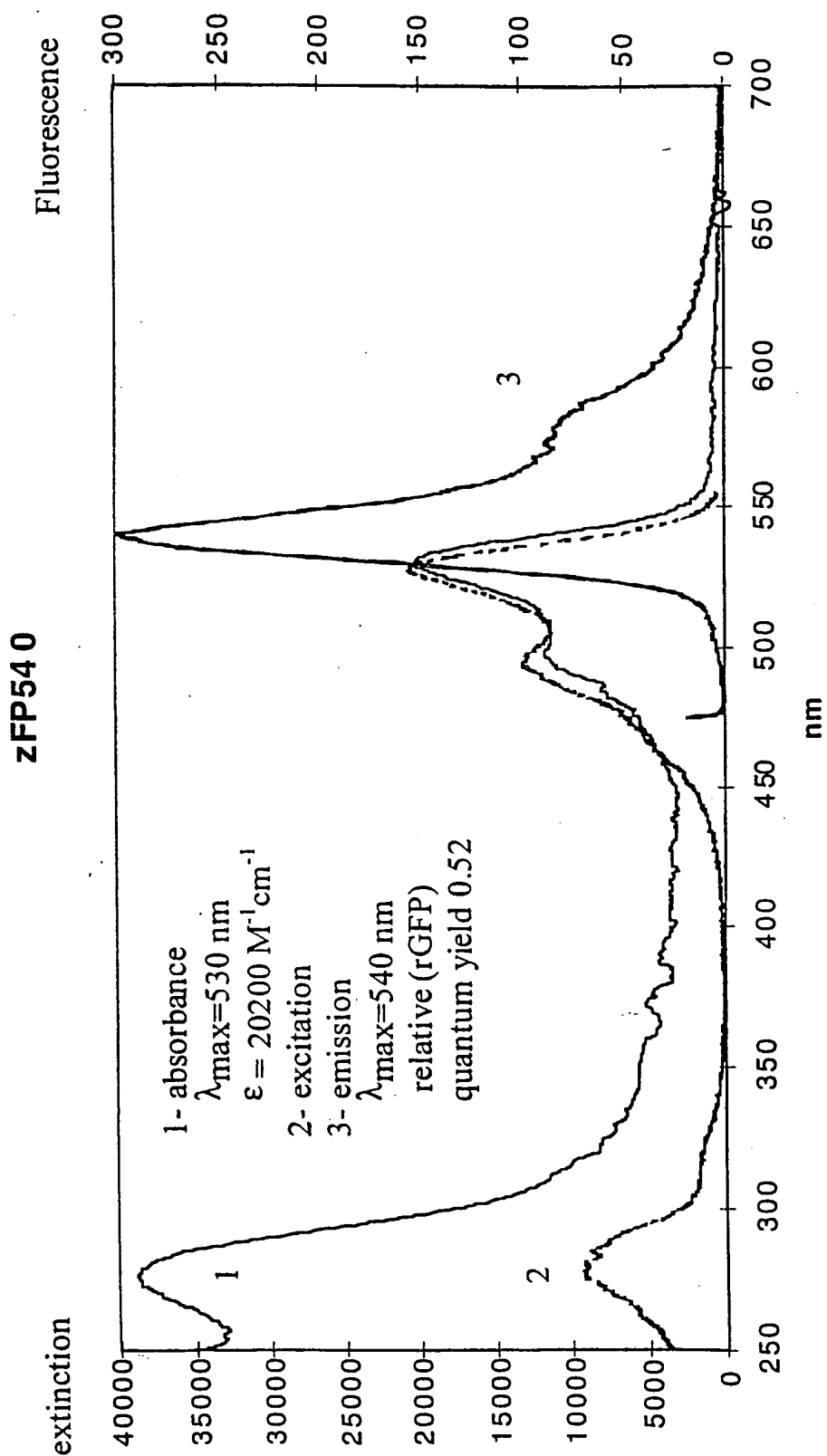
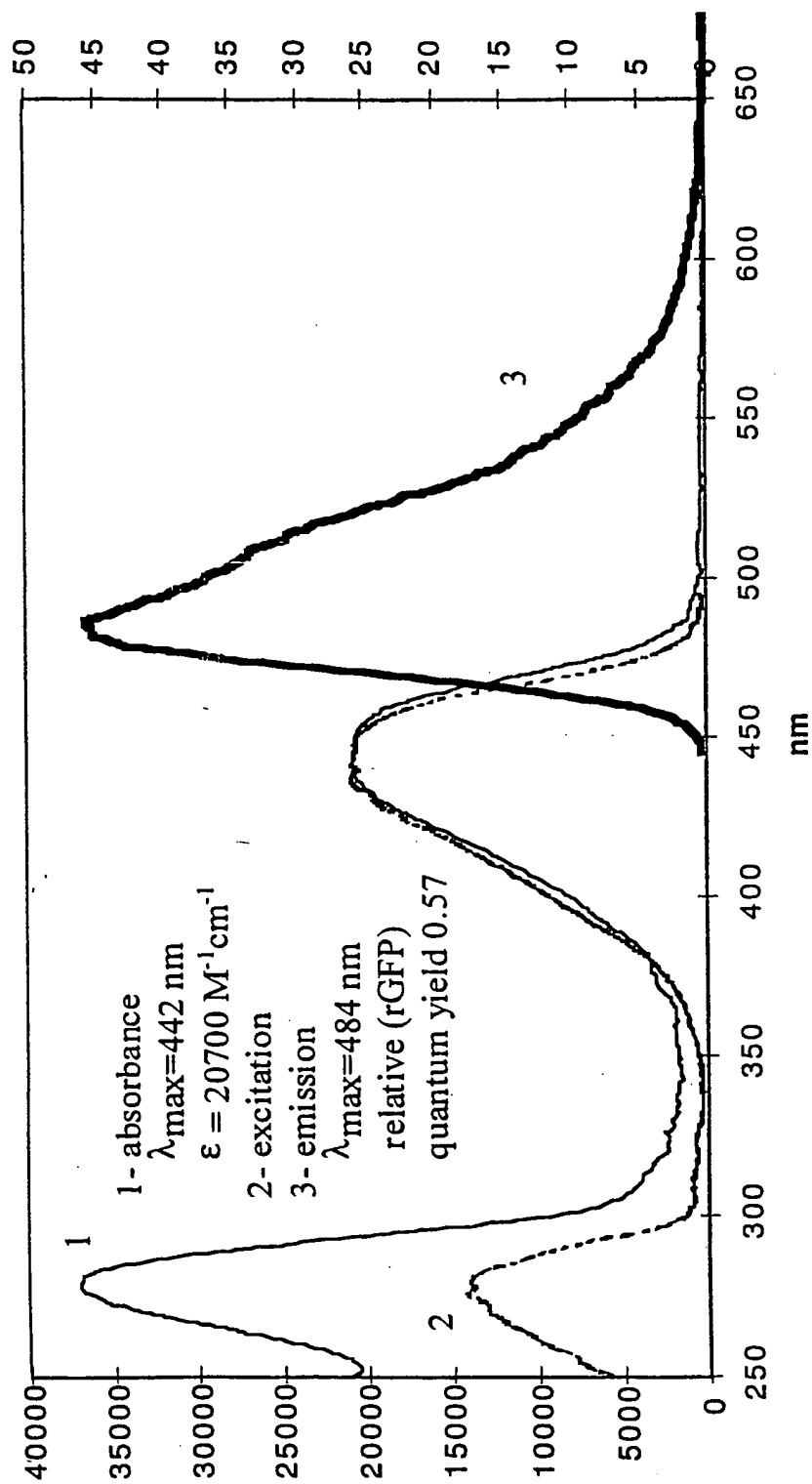


FIG. 6

dsFP484**FIG. 7**

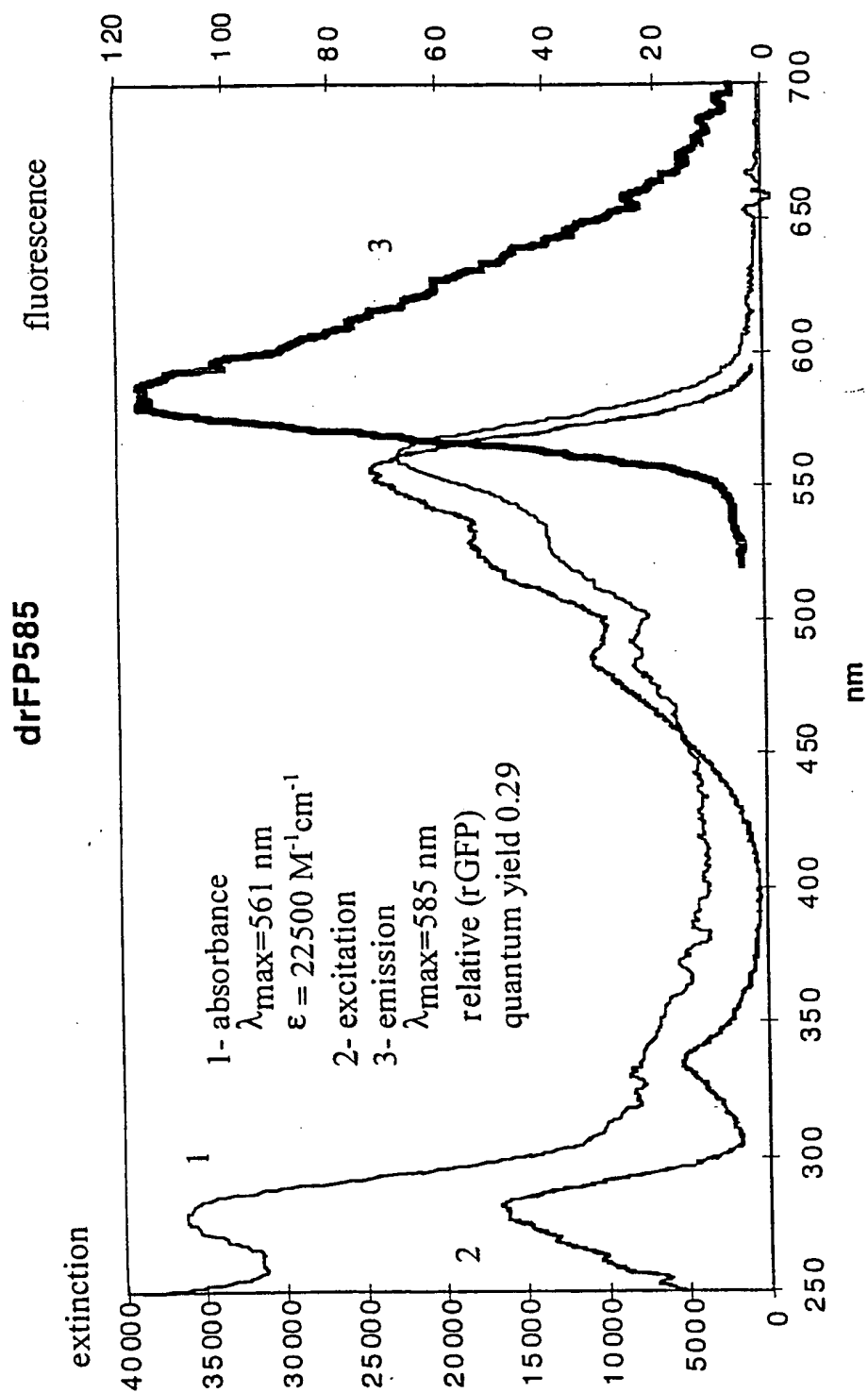


FIG. 8

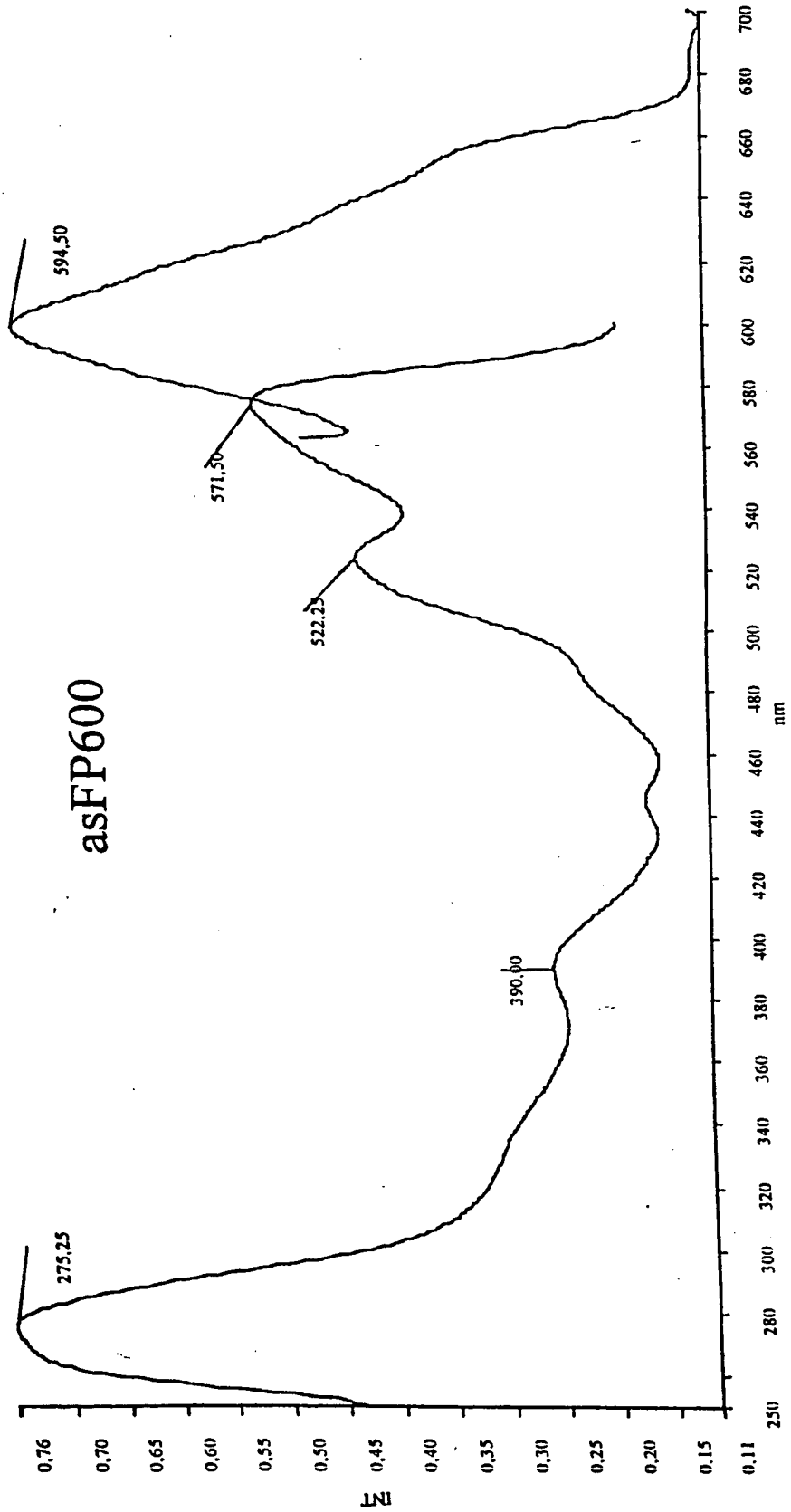


FIG. 9

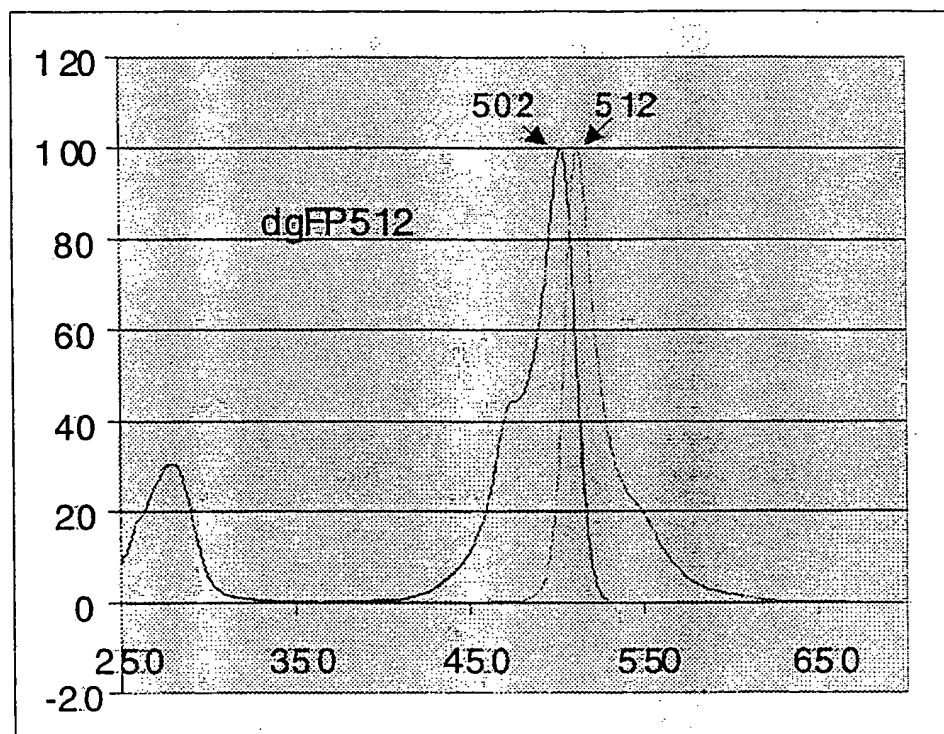


Fig. 10

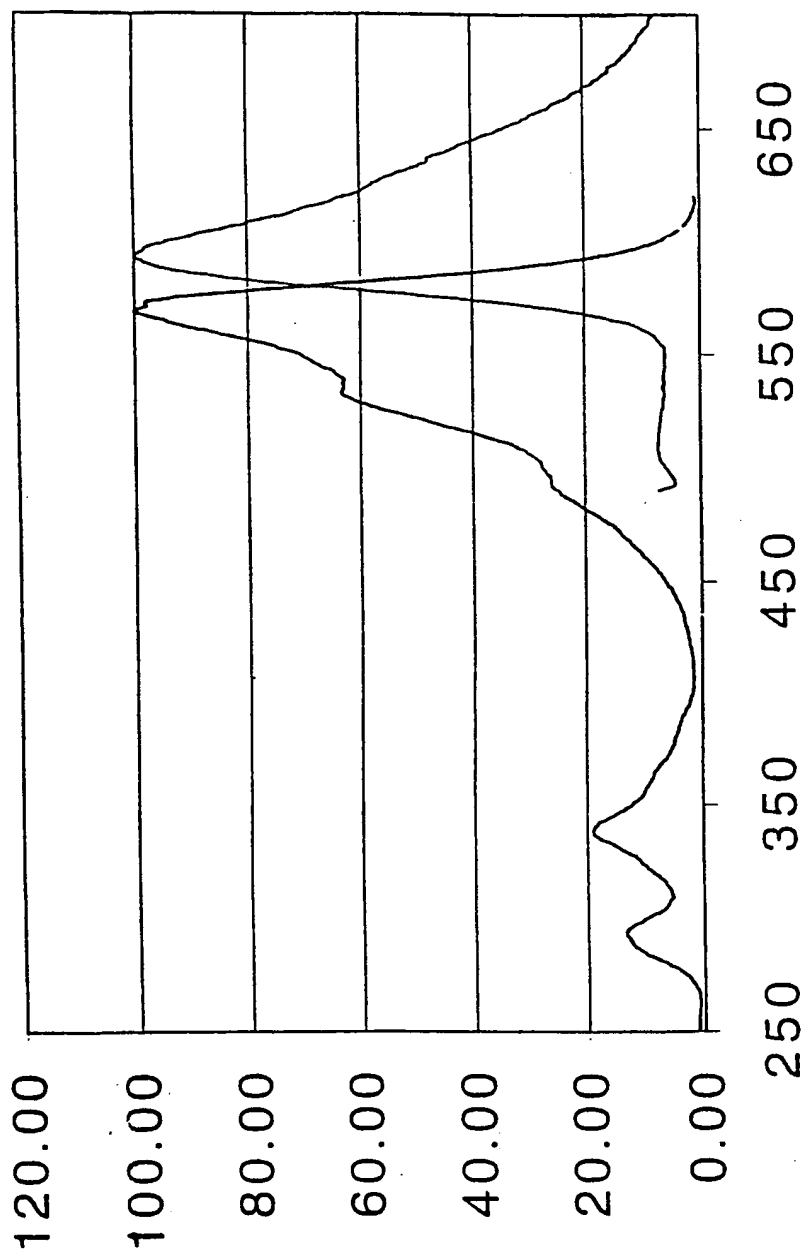


FIG. 11

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
 Labas, Yulii A.
 Matz, Mikhail V.
 5 Fradkov, Arcady F.
 <120> Fluorescent proteins from non-bioluminescent
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 proteins and uses thereof
 <130> D6196PCT
 10 <141> 1999-12-10
 <150> 09/210,330
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 5
 10
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 20 protein; n at position 12 represents any of the
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 5
 35
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30		<i>Anemonia majano</i>
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gtcaggcata ctggtaggat . 20

	<210>	22
35	<211>	21

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Discosoma striata

<400> 28

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19

5 <210> 29

<211> 17

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma striata

<400> 29

ctgcacaacg ggtccat

17

15

<210> 30

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<212> DNA

<213> artificial sequence

20 <220>

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Anemonia sulcata

<400> 30

25 cctctatctt catttcctgc

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<210> 31

<211> 20

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30 <213> artificial sequence

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<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata

35 <400> 31

tatcttcatt tcttgctac

20

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	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma sp. "magenta"</i>	
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	<210>	33	
	<211>	19	
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	<221>	primer_bind	
	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma sp. "magenta"</i>	
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25	<211>	22	
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	<221>	primer_bind	
30	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma sp. "green"</i>	
	<400>	34	
	ccctcagcaa tccatcacgt tc		22
35	<210>	35	
	<211>	20	
	<212>	DNA	

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 <223> gene-specific primer used for 5'-RACE for
 5 *Discosoma* sp. "green"
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 10 <210> 36
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 15 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia majano*
 <400> 36
 acatggatcc gctctttcaa acaagtttat c 31
 20 <210> 37
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 <212> DNA
 <213> artificial sequence
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 25 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Anemonia majano*
 <400> 37
 tagtactcga gcttattcgt atttcagtga aatc 34
 30
 <210> 38
 <211> 29
 <212> DNA
 <213> artificial sequence
 35 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region

of nFPs from *Clavularia sp.*

<400> 38

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29

5 <210> 39

<211> 28

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Clavularia sp.*

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28

15

<210> 40

<211> 31

<212> DNA

<213> artificial sequence

20 <220>

<221> primer_bind

<223> downstream primer used to obtain full coding
region of nFPs from *Clavularia sp.*

<400> 40

25 tagtactcga gcaacacaaa ccctcagaca a

31

<210> 41

<211> 28

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Zoanthus sp.*

35 <400> 41

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28

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	<223>	downstream primer used to obtain full coding region of nFPs from <i>Zoanthus</i> sp.	
10	<400>	42	
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	<210>	43	
	<211>	31	
15	<212>	DNA	
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	<220>		
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	<223>	upstream primer used to obtain full coding region of nFPs from <i>Discosoma</i> sp. "red"	
20	<400>	43	
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	<210>	44	
25	<211>	29	
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	<220>		
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30	<223>	downstream primer used to obtain full coding region of nFPs from <i>Discosoma</i> sp. "red"	
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35	<210>	45	
	<211>	28	
	<212>	DNA	

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 5 of nFPs from *Discosoma striata*
 <400> 45
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 <210> 46
 10 <211> 28
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 <213> artificial sequence
 <220>
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 15 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma striata*
 <400> 46
 tagcgagctc tatcatgcct cgtcacct 28

 20 <210> 47
 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 25 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia sulcata*
 <400> 47
 acatggatcc gcttcctttt taaagaagac t 31
 30
 <210> 48
 <211> 28
 <212> DNA
 <213> artificial sequence
 35 <220>
 <221> primer_bind
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region of nFPs from *Anemonia sulcata*

<400> 48

tagtactcga gtccttggga gcggcttg 28

5 <210> 49

<211> 30

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Discosoma sp. "magenta"*

<400> 49

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15 <210> 50

<211> 26

<212> DNA

<213> artificial sequence

<220>

20 <221> primer_bind

<223> downstream primer used to obtain full coding
region of nFPs from *Discosoma sp. "magenta"*

<400> 50

25 tagtactcga ggccattacg ctaatc 26

<210> 51

<211> 31

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Discosoma sp. "green"*

35 <400> 51

acatggatcc agtgcactta aagaagaaat g 31

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25	<212>	PRT	
	<213>	<i>Aequorea victoria</i>	
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	<400>	54	
30	Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu		
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	Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser		
		20	25 30
	Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys		
35		35	40 45
	Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu		
		50	55 60

	Val	Thr	Thr	Phe	Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro
					65					70					75
	Asp	His	Met	Lys	Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu
					80					85					90
5	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn
					95					100					105
	Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val
					110					115					120
	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn
10					125					130					135
	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val
					140					145					150
	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe
					155					160					165
15	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp
					170					175					180
	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	Leu	Leu
					185					190					195
	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp
20					200					205					210
	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	Thr
					215					220					225
	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Lys		
					230					235					
25															
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	<211>				229										
	<212>				PRT										
	<213>				<i>Anemonia majano</i>										
30	<220>														
	<223>				amino acid sequence of amFP486										
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	Met	Ala	Leu	Ser	Asn	Lys	Phe	Ile	Gly	Asp	Asp	Met	Lys	Met	Thr
					5					10					15
35	Tyr	His	Met	Asp	Gly	Cys	Val	Asn	Gly	His	Tyr	Phe	Thr	Val	Lys
					20					25					30
	Gly	Glu	Gly	Asn	Gly	Lys	Pro	Tyr	Glu	Gly	Thr	Gln	Thr	Ser	Thr
					35					40					45

Phe Lys Val Thr Met Ala Asn Gly Gly Pro Leu Ala Phe Ser Phe
 50 55 60
 Asp Ile Leu Ser Thr Val Phe Lys Tyr Gly Asn Arg Cys Phe Thr
 65 70 75
 5 Ala Tyr Pro Thr Ser Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro
 80 85 90
 Asp Gly Met Ser Tyr Glu Arg Thr Phe Thr Tyr Glu Asp Gly Gly
 95 100 105
 Val Ala Thr Ala Ser Trp Glu Ile Ser Leu Lys Gly Asn Cys Phe
 10 110 115 120
 Glu His Lys Ser Thr Phe His Gly Val Asn Phe Pro Ala Asp Gly
 125 130 135
 Pro Val Met Ala Lys Lys Thr Thr Gly Trp Asp Pro Ser Phe Glu
 140 145 150
 15 Lys Met Thr Val Cys Asp Gly Ile Leu Lys Gly Asp Val Thr Ala
 155 160 165
 Phe Leu Met Leu Gln Gly Gly Gly Asn Tyr Arg Cys Gln Phe His
 170 175 180
 Thr Ser Tyr Lys Thr Lys Lys Pro Val Thr Met Pro Pro Asn His
 185 190 195
 20 Val Val Glu His Arg Ile Ala Arg Thr Asp Leu Asp Lys Gly Gly
 200 205 210
 Asn Ser Val Gln Leu Thr Glu His Ala Val Ala His Ile Thr Ser
 215 220 225
 25 Val Val Pro Phe

<210> 56
 <211> 266
 30 <212> PRT
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 <220>
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 <400> 56
 35 Met Lys Cys Lys Phe Val Phe Cys Leu Ser Phe Leu Val Leu Ala
 5 10 15
 Ile Thr Asn Ala Asn Ile Phe Leu Arg Asn Glu Ala Asp Phe Glu

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	Glu	Lys	Thr	Phe	Arg	Ile	Pro	Lys	Ala	Leu	Thr	Thr	Met	Gly	Val
		35		40		45									
	Ile	Lys	Pro	Asp	Met	Lys	Ile	Lys	Leu	Lys	Met	Glu	Gly	Asn	Val
5		50		55		60									
	Asn	Gly	His	Ala	Phe	Val	Ile	Glu	Gly	Glu	Gly	Glu	Gly	Lys	Pro
		65		70		75									
	Tyr	Asp	Gly	Thr	His	Thr	Leu	Asn	Leu	Glu	Val	Lys	Glu	Gly	Ala
		80		85		90									
10	Pro	Leu	Pro	Phe	Ser	Tyr	Asp	Ile	Leu	Ser	Asn	Ala	Phe	Gln	Tyr
		95		100		105									
	Gly	Asn	Arg	Ala	Leu	Thr	Lys	Tyr	Pro	Asp	Asp	Ile	Ala	Asp	Tyr
		110		115		120									
	Phe	Lys	Gln	Ser	Phe	Pro	Glu	Gly	Tyr	Ser	Trp	Glu	Arg	Thr	Met
15		125		130		135									
	Thr	Phe	Glu	Asp	Lys	Gly	Ile	Val	Lys	Val	Lys	Ser	Asp	Ile	Ser
		140		145		150									
	Met	Glu	Glu	Asp	Ser	Phe	Ile	Tyr	Glu	Ile	Arg	Phe	Asp	Gly	Met
		155		160		165									
20	Asp	Phe	Pro	Pro	Asn	Gly	Pro	Val	Met	Gln	Lys	Lys	Thr	Leu	Lys
		170		175		180									
	Trp	Glu	Pro	Ser	Thr	Glu	Ile	Met	Tyr	Val	Arg	Asp	Gly	Val	Leu
		185		190		195									
	Val	Gly	Asp	Ile	Ser	His	Ser	Leu	Leu	Leu	Glu	Gly	Gly	Gly	His
25		200		205		210									
	Tyr	Arg	Cys	Asp	Phe	Lys	Ser	Ile	Tyr	Lys	Ala	Lys	Lys	Val	Val
		215		220		225									
	Lys	Leu	Pro	Asp	Tyr	His	Phe	Val	Asp	His	Arg	Ile	Glu	Ile	Leu
		230		235		240									
30	Asn	His	Asp	Lys	Asp	Tyr	Asn	Lys	Val	Thr	Leu	Tyr	Glu	Asn	Ala
		245		250		255									
	Val	Ala	Arg	Tyr	Ser	Leu	Leu	Pro	Ser	Gln	Ala				
		260		265											
35	<210>	57													
	<211>	230													
	<212>	PRT													

<213> *Zoanthus sp.*
 <220>
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 <400> 57

5	Ala	Gln	Ser	Lys	His	Gly	Leu	Thr	Lys	Glu	Met	Thr	Met	Lys	Tyr	
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	Arg	Met	Glu	Gly	Cys	Val	Asp	Gly	His	Lys	Phe	Val	Ile	Thr	Gly	
					20					25					30	
	Glu	Gly	Ile	Gly	Tyr	Pro	Phe	Lys	Gly	Lys	Gln	Ala	Ile	Asn	Leu	
10					35					40					45	
	Cys	Val	Val	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Glu	Asp	Ile	Leu	
					50					55					60	
	Ser	Ala	Ala	Phe	Asn	Tyr	Gly	Asn	Arg	Val	Phe	Thr	Glu	Tyr	Pro	
					65					70					75	
15	Gln	Asp	Ile	Val	Asp	Tyr	Phe	Lys	Asn	Ser	Cys	Pro	Ala	Gly	Tyr	
					80					85					90	
	Thr	Trp	Asp	Arg	Ser	Phe	Leu	Phe	Glu	Asp	Gly	Ala	Val	Cys	Ile	
					95					100					105	
	Cys	Asn	Ala	Asp	Ile	Thr	Val	Ser	Val	Glu	Glu	Asn	Cys	Met	Tyr	
20					110					115					120	
	His	Glu	Ser	Lys	Phe	Tyr	Gly	Val	Asn	Phe	Pro	Ala	Asp	Gly	Pro	
					125					130					135	
	Val	Met	Lys	Lys	Met	Thr	Asp	Asn	Trp	Glu	Pro	Ser	Cys	Glu	Lys	
					140					145					150	
25	Ile	Ile	Pro	Val	Pro	Lys	Gln	Gly	Ile	Leu	Lys	Gly	Asp	Val	Ser	
					155					160					165	
	Met	Tyr	Leu	Leu	Leu	Lys	Asp	Gly	Gly	Arg	Leu	Arg	Cys	Gln	Phe	
					170					175					180	
	Asp	Thr	Val	Tyr	Lys	Ala	Lys	Ser	Val	Pro	Arg	Lys	Met	Pro	Asp	
30					185					190					195	
	Trp	His	Phe	Ile	Gln	His	Lys	Leu	Thr	Arg	Glu	Asp	Arg	Ser	Asp	
					200					205					210	
	Ala	Lys	Asn	Gln	Lys	Trp	His	Leu	Thr	Glu	His	Ala	Ile	Ala	Ser	
					215					220					225	
35	Gly	Ser	Ala	Leu	Pro											
					230											

<210> 58
 <211> 230
 <212> PRT
 5 <213> *Zoanthus sp.*
 <220>
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 <400> 58

Met Ala His Ser Lys His Gly Leu Lys Glu Glu Met Thr Met Lys
 10 5 10 15
 Tyr His Met Glu Gly Cys Val Asn Gly His Lys Phe Val Ile Thr
 20 25 30
 Gly Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Thr Ile Asn
 35 40 45
 15 Leu Cys Val Ile Glu Gly Gly Pro Leu Pro Phe Ser Glu Asp Ile
 50 55 60
 Leu Ser Ala Gly Phe Lys Tyr Gly Asp Arg Ile Phe Thr Glu Tyr
 65 70 75
 Pro Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly
 20 80 85 90
 Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
 95 100 105
 Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr
 110 115 120
 25 His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro
 125 130 135
 Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys
 140 145 150
 Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
 30 155 160 165
 Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe
 170 175 180
 Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu
 185 190 195
 35 Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp
 200 205 210
 Ala Lys Asn Gln Lys Trp Gln Leu Thr Glu His Ala Ile Ala Phe
 215 220 225

Pro Ser Ala Leu Ala

230

5 <210> 59
 <211> 232
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 <220>
 10 <223> amino acid sequence of dsFP483
 <400> 59

Met Ser Cys Ser Lys Ser Val Ile Lys Glu Glu Met Leu Ile Asp
 5 10 15
 Leu His Leu Glu Gly Thr Phe Asn Gly His Tyr Phe Glu Ile Lys
 15 20 25 30
 Gly Lys Gly Lys Gly Gln Pro Asn Glu Gly Thr Asn Thr Val Thr
 35 40 45
 Leu Glu Val Thr Lys Gly Gly Pro Leu Pro Phe Gly Trp His Ile
 50 55 60
 20 Leu Cys Pro Gln Phe Gln Tyr Gly Asn Lys Ala Phe Val His His
 65 70 75
 Pro Asp Asn Ile His Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly
 80 85 90
 Tyr Thr Trp Glu Arg Ser Met His Phe Glu Asp Gly Gly Leu Cys
 25 95 100 105
 Cys Ile Thr Asn Asp Ile Ser Leu Thr Gly Asn Cys Phe Tyr Tyr
 110 115 120
 Asp Ile Lys Phe Thr Gly Leu Asn Phe Pro Pro Asn Gly Pro Val
 125 130 135
 30 Val Gln Lys Lys Thr Thr Gly Trp Glu Pro Ser Thr Glu Arg Leu
 140 145 150
 Tyr Pro Arg Asp Gly Val Leu Ile Gly Asp Ile His His Ala Leu
 155 160 165
 Thr Val Glu Gly Gly Gly His Tyr Ala Cys Asp Ile Lys Thr Val
 35 170 175 180
 Tyr Arg Ala Lys Lys Ala Ala Leu Lys Met Pro Gly Tyr His Tyr
 185 190 195

Val Asp Thr Lys Leu Val Ile Trp Asn Asn Asp Lys Glu Phe Met
 200 205 210
 Lys Val Glu Glu His Glu Ile Ala Val Ala Arg His His Pro Phe
 215 220 225
 5 Tyr Glu Pro Lys Lys Asp Lys
 230
 <210> 60
 <211> 225
 10 <212> PRT
 <213> *Discosoma* sp. "red"
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 5 10 15
 Val Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Glu
 20 25 30
 Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Asn Thr Val Lys
 20 35 40 45
 Leu Lys Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp Ile
 50 55 60
 Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His
 65 70 75
 25 Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly
 80 85 90
 Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val
 95 100 105
 Thr Val Thr Gln Asp Ser Ser Leu Gln Asp Gly Cys Phe Ile Tyr
 30 110 115 120
 Lys Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val
 125 130 135
 Met Gln Lys Lys Thr Met Gly Trp Glu Ala Ser Thr Glu Arg Leu
 140 145 150
 35 Tyr Pro Arg Asp Gly Val Leu Lys Gly Glu Ile His Lys Ala Leu
 155 160 165
 Lys Leu Lys Asp Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
 170 175 180

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Tyr Met Ala Lys Lys Pro Val Gln Leu Pro Gly Tyr Tyr Tyr Val
--          185                      190                      195
Asp Ser Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr Thr Ile
          200                      205                      210
5 Val Glu Gln Tyr Glu Arg Thr Glu Gly Arg His His Leu Phe Leu
          215                      220                      225

      <210>      61
      <211>      232
10      <212>      PRT
      <213>      Anemonia sulcata
      <220>
      <223>      amino acid sequence of asFP600
      <400>      61

15 Met Ala Ser Phe Leu Lys Lys Thr Met Pro Phe Lys Thr Thr Ile
          5                      10                      15
Glu Gly Thr Val Asn Gly His Tyr Phe Lys Cys Thr Gly Lys Gly
          20                      25                      30
Glu Gly Asn Pro Phe Glu Gly Thr Gln Glu Met Lys Ile Glu Val
20          35                      40                      45
Ile Glu Gly Gly Pro Leu Pro Phe Ala Phe His Ile Leu Ser Thr
          50                      55                      60
Ser Cys Met Tyr Gly Ser Lys Thr Phe Ile Lys Tyr Val Ser Gly
          65                      70                      75
25 Ile Pro Asp Tyr Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp
          80                      85                      90
Glu Arg Thr Thr Thr Tyr Glu Asp Gly Gly Phe Leu Thr Ala His
          95                      100                     105
Gln Asp Thr Ser Leu Asp Gly Asp Cys Leu Val Tyr Lys Val Lys
30          110                     115                     120
Ile Leu Gly Asn Asn Phe Pro Ala Asp Gly Pro Val Met Gln Asn
          125                     130                     135
Lys Ala Gly Arg Trp Glu Pro Ala Thr Glu Ile Val Tyr Glu Val
          140                     145                     150
35 Asp Gly Val Leu Arg Gly Gln Ser Leu Met Ala Leu Lys Cys Pro
          155                     160                     165
Gly Gly Arg His Leu Thr Cys His Leu His Thr Thr Tyr Arg Ser
          170                     175                     180

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Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu
 185 190 195
 Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys
 200 205 210
 5 Tyr Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala
 215 220 225
 Pro Ser Lys Leu Gly His Asn
 230

10 <210> 62
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 <212> PRT
 <213> *Discosoma* sp. "green"
 <220>
 15 <223> amino acid sequence of dgFP512
 <400> 62

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 Gly Val Val Asn Gly Leu Pro Phe Lys Ile Arg Gly Asp Gly Lys
 20 20 25 30
 Gly Lys Pro Tyr Gln Gly Ser Gln Glu Leu Thr Leu Thr Val Val
 35 40 45
 Lys Gly Gly Pro Leu Pro Phe Ser Tyr Asp Ile Leu Thr Thr Met
 50 55 60
 25 Phe Gln Tyr Gly Asn Arg Ala Phe Val Asn Tyr Pro Glu Asp Ile
 65 70 75
 Pro Asp Ile Phe Lys Gln Thr Cys Ser Gly Pro Asn Gly Gly Tyr
 80 85 90
 Ser Trp Gln Arg Thr Met Thr Tyr Glu Asp Gly Gly Val Cys Thr
 30 95 100 105
 Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp
 110 115 120

Ile His Phe Met Gly Ala Asn Phe Pro Leu Asp Gly Pro Val Met
 125 130 135
 Gln Lys Arg Thr Met Lys Trp Glu Pro Ser Thr Glu Ile Met Phe
 140 145 150
 5 Glu Arg Asp Gly Met Leu Arg Gly Asp Ile Ala Met Ser Leu Leu
 155 160 165
 Leu Lys Gly Gly Gly His Tyr Arg Cys Asp Phe Glu Thr Ile Tyr
 170 175 180
 Lys Pro Asn Lys Val Val Lys Met Pro Asp Tyr His Phe Val Asp
 10 185 190 195
 His Cys Ile Glu Ile Thr Ser Gln Gln Asp Tyr Tyr Asn Val Val
 200 205 210
 Glu Leu Thr Glu Val Ala Glu Ala Arg Tyr Ser Ser Leu Glu Lys
 215 220 225
 15 Ile Gly Lys Ser Lys Ala
 230

 <210> 63
 <211> 235
 20 <212> PRT
 <213> *Discosoma* sp. "magenta"
 <220>
 <223> amino acid sequence of dmFP592
 <400> 63
 25 Met Ser Cys Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys
 5 10 15
 Val Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Lys
 20 25 30
 Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys
 30 35 40 45

	Leu Met Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile		
	50	55	60
	Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His		
	65	70	75
5	Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly		
	80	85	90
	Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val		
	100	105	110
	Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly Cys Phe Ile Tyr		
10	115	120	125
	Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val		
	130	135	140
	Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu Arg Leu		
	145	150	155
15	Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu		
	160	165	170
	Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile		
	175	180	185
	Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr		
20	190	195	200
	Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr		
	205	210	215
	Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe		
	220	225	230
25	Ile Lys Pro Leu Gln		
	235		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29405

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; C07K 14/435

US CL : 435/6, 69.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOs.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 FEBRUARY 2000

Date of mailing of the international search report

02 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GABRIELE ELISABETH BUGAISKY

Telephone No. (703) 308-0196